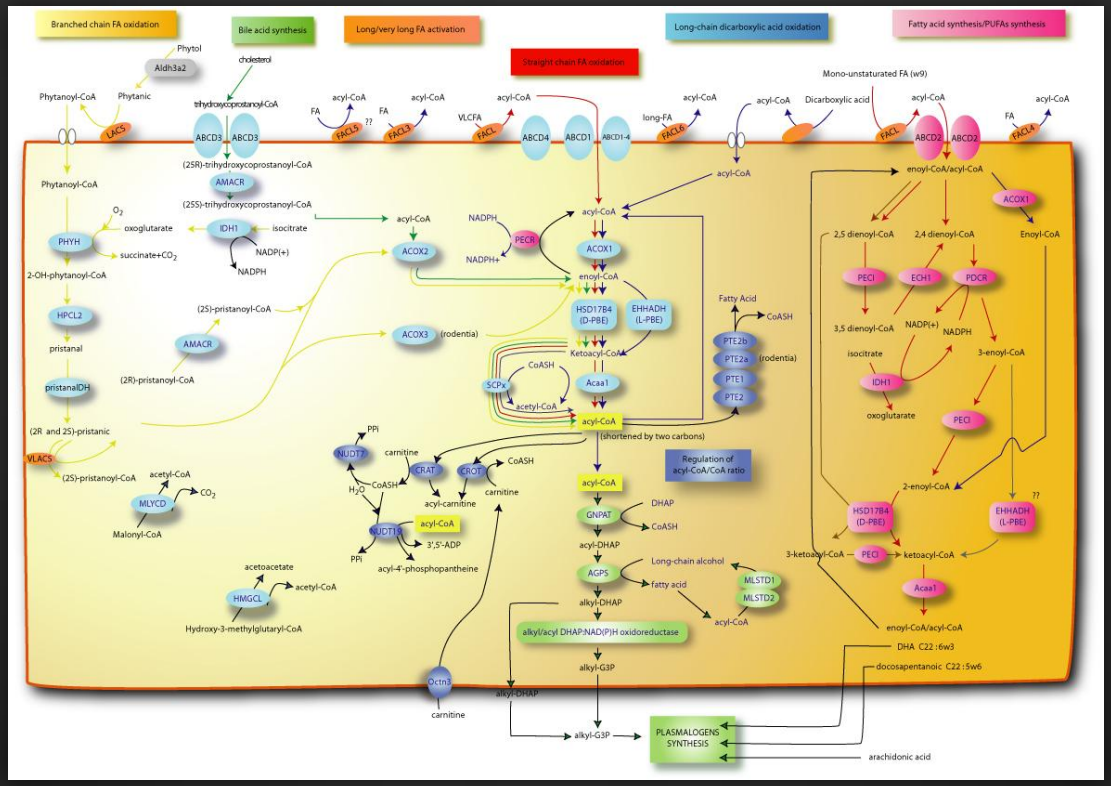
Synthetic Biological Systems

1. Metabolic Engineering with Synthetic Biology

What is Metabolic Engineering?

"Metabolic engineering is the practice of optimizing genetic and regulatory processes within cells to increase the cells' production of a certain substance" Wikipedia



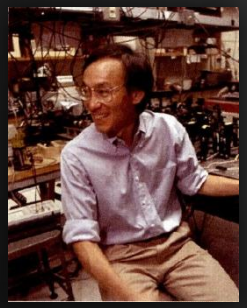
Metabolic engineering for biosynthesis

An attractive alternative to chemical synthesis

- Generate fuels from renewable resources
- Convert biomass into chemicals – both bulk and speciality
- Produce therapeutic compounds that are a chemical challenge
- Tap into nature's huge diversity

A 21st century goldmine

- Major push by US Department of Energy – "12 value-added compounds"
- Huge investments from BP, Chevron, Bill Gates
- Green alternative to the petrochemical industry



Lecture Content

In this lecture we'll learn about:

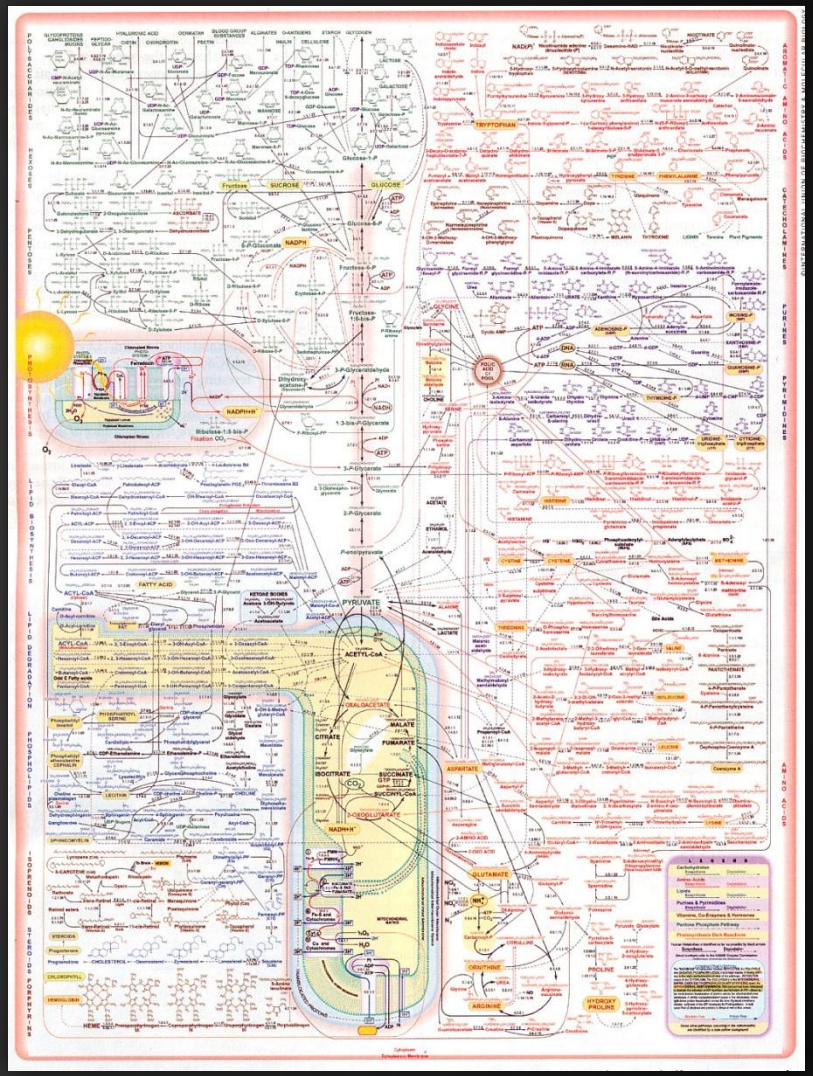
1. The complexity of metabolism in a cell and the major challenges in metabolic engineering
2. What synthetic biology offers for metabolic engineering
3. The data and models associated with metabolic engineering

4. A major project to engineer the synthesis of a crucial anti-malarial in both *E.coli* and yeast cells
5. Engineering bacteria to produce biopetrol from sunlight and CO2
6. Engineering bacteria to turn plant waste into diesel biofuels

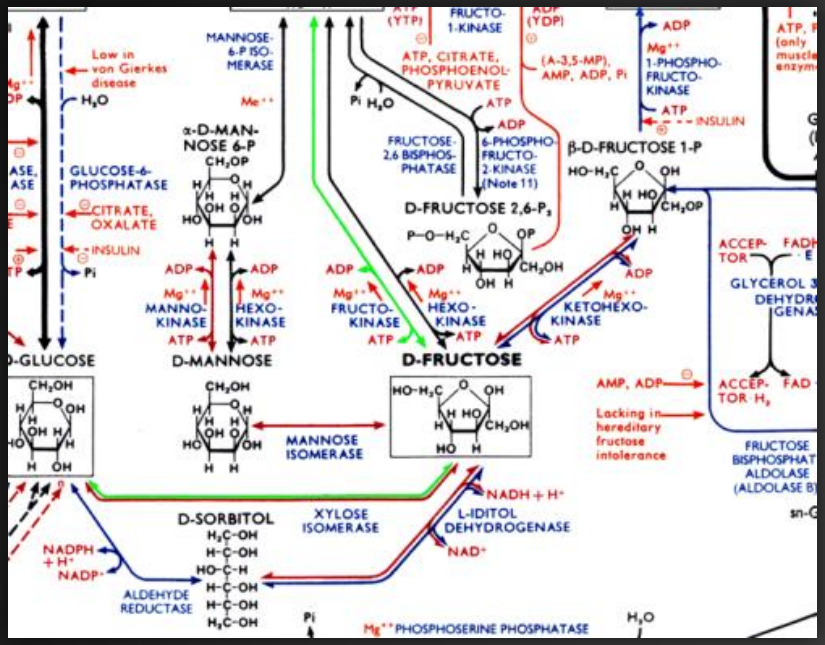
7. Novel synthetic biology methods to optimise metabolic pathways
8. The future for synthetic biology and metabolic engineering

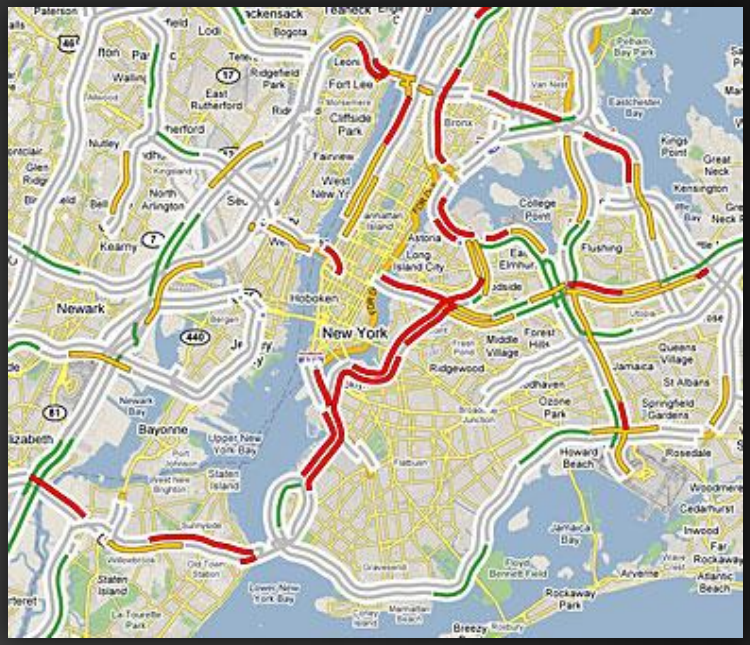
Cellular metabolism is a complex network

- Cellular metabolism is a complex inter-dependent network
- The metabolome is all the small molecules of a cell
- Metabolic networks are defined by pathways
- Flux is the rate of turnover of molecules through a pathway
- Flux is regulated by the enzymes in a pathway



Metabolic engineering is like managing traffic





- Carbons and other atoms are the people
- Metabolites are their location
- Enzymes are the roads and railways they travel on

Synthetic biology for metabolic engineering

Synthetic Biology offers:

1. Predictable, designed genetic engineering
2. Regulation of gene expression
3. Insertion of new genes and new functions
4. Scalability – the addition or modification of many genes

This gives metabolic engineering:

1. Engineering by design
2. Control of enzyme levels, and so control of flux
3. Synthesis of new products
4. Assembly of whole new pathways

Basic metabolic engineering: change gene expression

- Increase the expression of enzymes involved in synthesis
  - add extra copies of enzyme gene into the cell
  - over-express gene using strong, regulated promoter
- Remove (knock-out) enzymes that takeaway from synthesis
  - use homologous recombination to delete host genes
- Careful balancing act – toxic precursors, growth rates
  - accumulation of products inhibits pathway production
  - many intermediates are toxic at high levels
  - cell global metabolism is always effected
    - resources must be taken from somewhere
    - slower growth is less yield

Further metabolic engineering: change the genes

- Modify the enzymes

  remove regulatory sub-units, change cell localisation

- Add enzymes from across nature – heterologous pathways

  400,000 known proteins and millions unknown

- Design new enzymes from scratch

  de novo design of active sites

- Directed evolution

  mutate the enzymes, select improvements, repeat

Measuring the results of metabolic engineering

- GC Mass Spec
  - Always on CSI

  Gas liquid chromatography plus mass spectrometry
- Growth rate
- Radio-labelled atoms
- Enzyme assays

- Gene expression data

  mRNA levels: cDNA array chips / sequencing

  protein levels: proteomics / MALDI-TOF

Predicting metabolic engineering

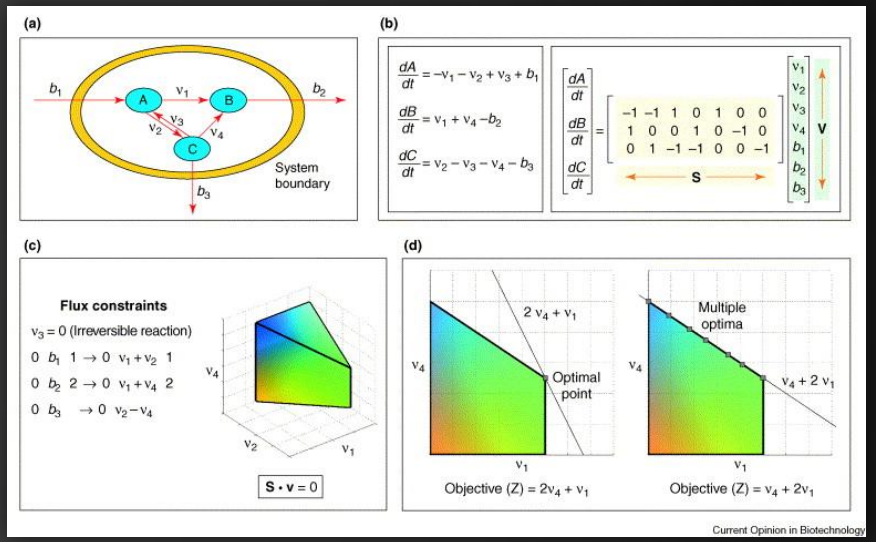
How to avoid bottlenecks, toxins, and negative feedback?

- Flux-balance analysis (FBA)

Mathematical analysis of the metabolic network under pertubations

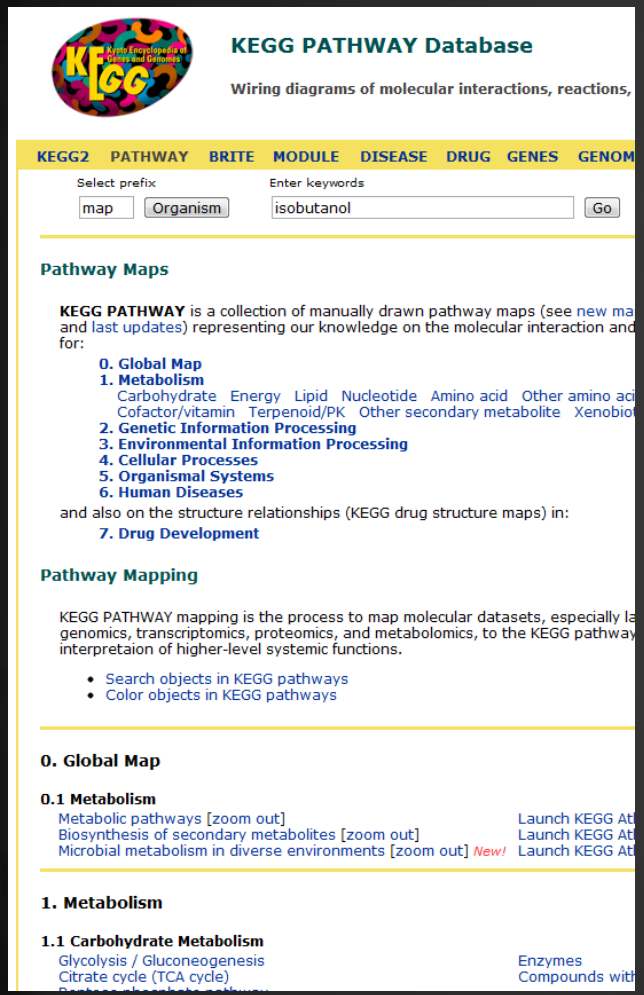
Model built using GC-MS data under different conditions

Most metabolism across the cell relies on the levels of a few chemicals: ATP/ADP, NAD+/NADH, NADP+/NADPH, Acyl-CoAs, TCA cycle



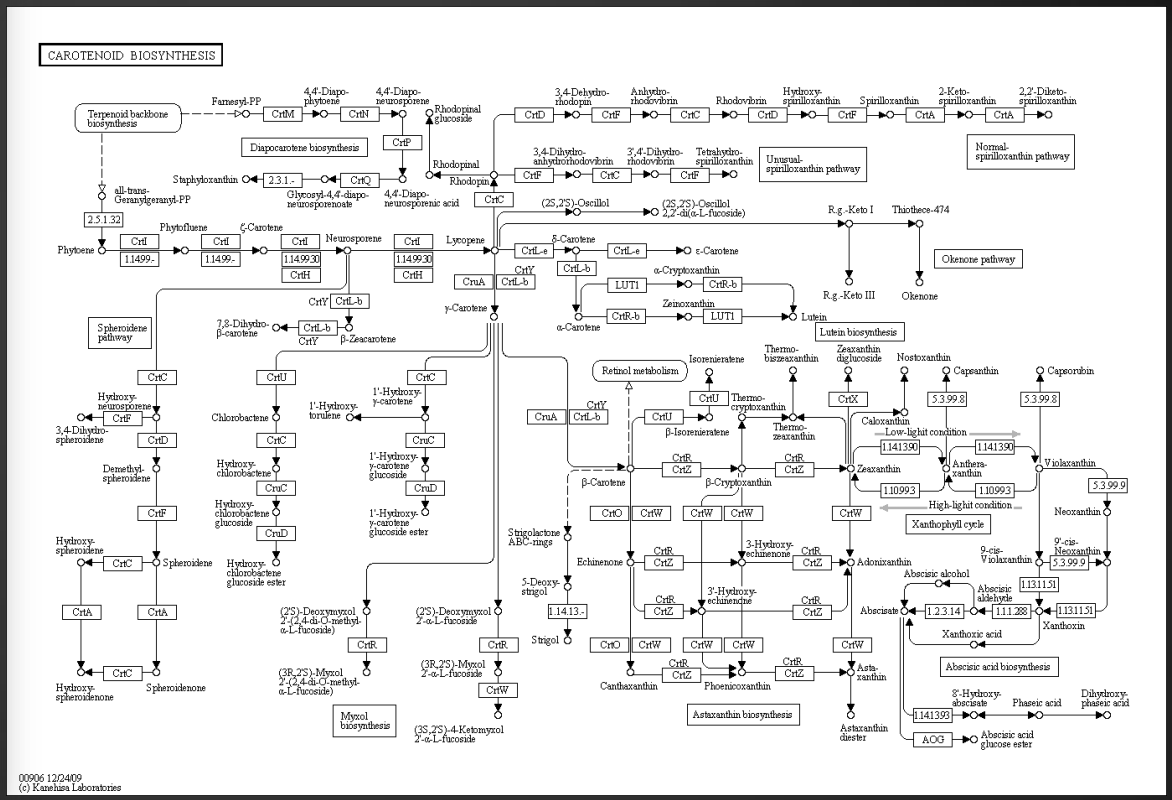
Next Gen: FBA with gene expression = Metabolomics + Systems Biology

Bioinformatics for Metabolic Engineering

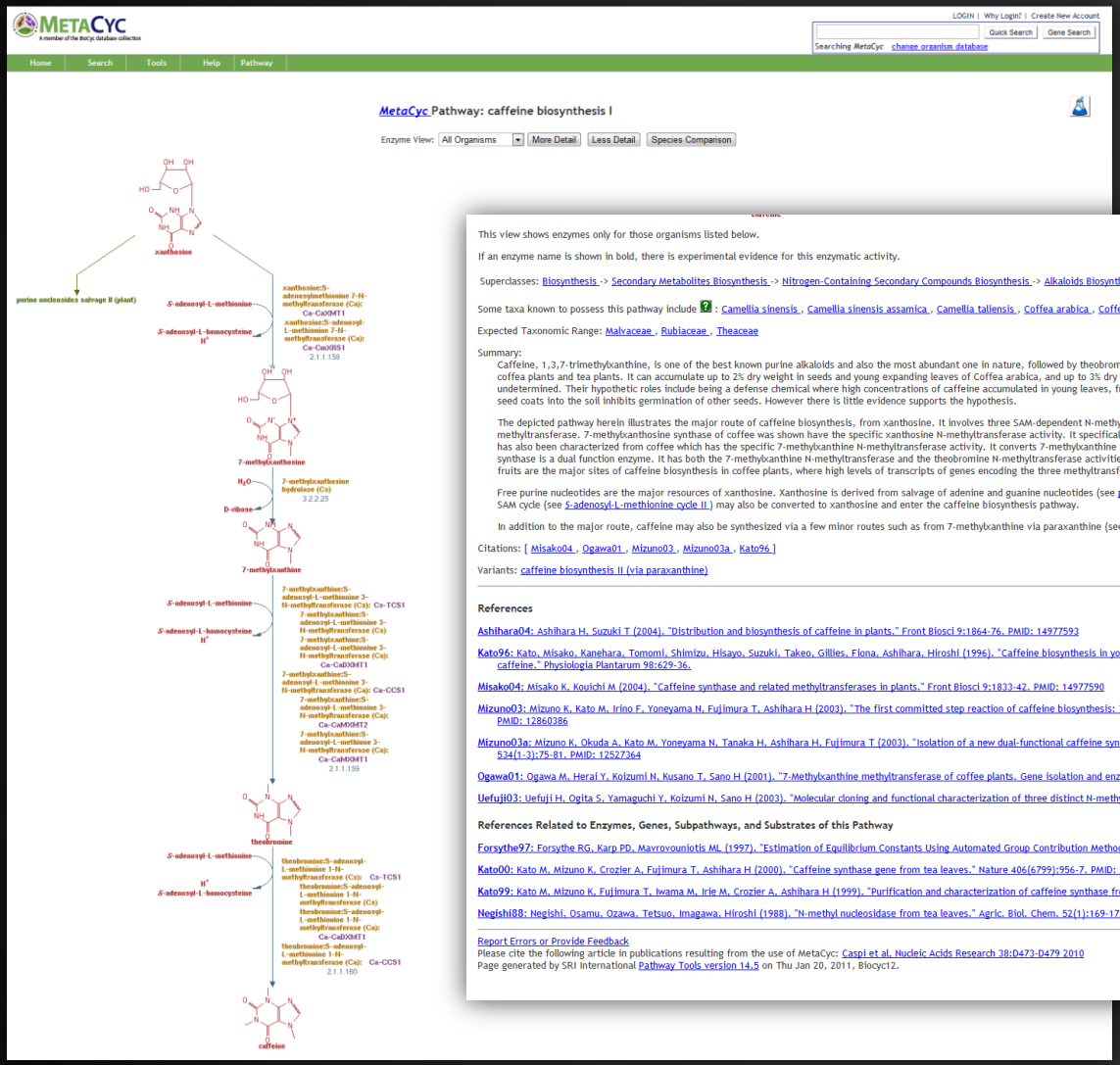


Kegg Pathway has thousands of annotated pathways

Example: Carotenoids – e.g. Lycopene, Carotene



Bioinformatics for Metabolic Engineering



MetaCyc

Example: Caffeine synthesis

This view shows enzymes only for those organisms listed below.

If an enzyme name is shown in bold, there is experimental evidence for this enzymatic activity.

Superclasses: Biosynthesis -> Secondary Metabolites Biosynthesis -> Nitrogen-Containing Secondary Compounds Biosynthesis -> Alkaloids Biosynthesis -> Purine Alkaloids Biosynthesis -> Caffeine Biosynthesis

Some taxa known to possess this pathway include : Camellia sinensis , Camellia sinensis assamica , Camellia taliensis , Coffea arabica , Coffea canephora

Expected Taxonomic Range: Malvaceae , Rubiaceae , Theaceae

Summary:

Caffeine, 1,3,7-trimethylxanthine, is one of the best known purine alkaloids and also the most abundant one in nature, followed by theobromine, the immediate precursor of caffeine (3,7-dimethylxanthine) [Ashihara04]. Caffeine presents in high concentrations in many coffea plants and tea plants. It can accumulate up to 2% dry weight in seeds and young expanding leaves of Coffea arabica, and up to 3% dry weight in young leaves of tea (Camellia sinensis). The exact physiological role of endogenous purine alkaloids in plants is undetermined. Their hypothetic roles include being a defense chemical where high concentrations of caffeine accumulated in young leaves, fruits and flower buds help protect these soft tissues from predators, or being an autotoxic chemical where caffeine released from seed coats into the soil inhibits germination of other seeds. However there is little evidence supports the hypothesis.

The depicted pathway herein illustrates the major route of caffeine biosynthesis, from xanthosine. It involves three SAM-dependent N-methyltransferase activities, namely xanthosine N-methyltransferase, 7-methylxanthine N-methyltransferase, and theobromine N-methyltransferase. 7-methylxanthosine synthase of coffee was shown have the specific xanthosine N-methyltransferase activity. It specifically converts xanthosine to 7-methylxanthosine (the first methylation step). XMP is not an effective substrate. Theobromine synthase has also been characterized from coffee which has the specific 7-methylxanthine N-methyltransferase activity. It converts 7-methylxanthine to theobromine (the second methylation step). Caffeine synthase has been characterized from both coffee and tea. Caffeine synthase is a dual function enzyme. It has both the 7-methylxanthine N-methyltransferase and the theobromine N-methyltransferase activities. It converts 7-methylxanthine to theobromine and theobromine to caffeine, the second and the third methylation steps. Immature fruits are the major sites of caffeine biosynthesis in coffee plants, where high levels of transcripts of genes encoding the three methyltransferation activities are also found [Uefuji03].

Free purine nucleotides are the major resources of xanthosine. Xanthosine is derived from salvage of adenine and guanine nucleotides (see purine nucleosides salvage II (plant)). Newly synthesized IMP from de novo purine biosynthesis, and adenosine released from the SAM cycle (see S-adenosyl-L-methionine cycle II) may also be converted to xanthosine and enter the caffeine biosynthesis pathway.

In addition to the major route, caffeine may also be synthesized via a few minor routes such as from 7-methylxanthine via paraxanthine (see caffeine biosynthesis II (via paraxanthine)).

Citations: [Misako04 , Ogawa01 , Mizuno03 , Mizuno03a , Kato96]

Variants: caffeine biosynthesis II (via paraxanthine)

References

Ashihara04: Ashihara H, Suzuki T (2004). "Distribution and biosynthesis of caffeine in plants." Front Biosci 9:1864-76. PMID: 14977593

Kato96: Kato, Misako, Kanehara, Tomomi, Shimizu, Hisayo, Suzuki, Takeo, Gillies, Fiona, Ashihara, Hiroshi (1996). "Caffeine biosynthesis in young leaves of Camellia sinensis: in vitro studies on N-methyl transferase activity involved in the conversion of xanthosine to caffeine." Physiologia Plantarum 98:629-36.

Misako04: Misako K, Kouichi M (2004). "Caffeine synthase and related methyltransferases in plants." Front Biosci 9:1833-42. PMID: 14977590

Mizuno03: Mizuno K, Kato M, Irino F, Yoneyama N, Fujimura T, Ashihara H (2003). "The first committed step reaction of caffeine biosynthesis: 7-methylxanthosine synthase is closely homologous to caffeine synthases in coffee (Coffea arabica L.)." FEBS Lett 547(1-3):56-60. PMID: 12860386

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Ogawa01: Ogawa M, Herai Y, Koizumi N, Kusano T, Sano H (2001). "7-Methylxanthine methyltransferase of coffee plants. Gene isolation and enzymatic properties." J Biol Chem 276(11):8213-8. PMID: 11108716

Uefuji03: Uefuji H, Ogita S, Yamaguchi Y, Koizumi N, Sano H (2003). "Molecular cloning and functional characterization of three distinct N-methyltransferases involved in the caffeine biosynthetic pathway in coffee plants." Plant Physiol 132(1):372-80. PMID: 12746542

References Related to Enzymes, Genes, Subpathways, and Substrates of this Pathway

Forsythe97: Forsythe RG, Karp PD, Mavrovouniotis ML (1997). "Estimation of Equilibrium Constants Using Automated Group Contribution Methods." CABIOS 13(5):537-543.

Kato00: Kato M, Mizuno K, Crozier A, Fujimura T, Ashihara H (2000). "Caffeine synthase gene from tea leaves." Nature 406(6799):956-7. PMID: 10984041

Kato99: Kato M, Mizuno K, Fujimura T, Iwama M, Irie M, Crozier A, Ashihara H (1999). "Purification and characterization of caffeine synthase from tea leaves." Plant Physiol 120(2):579-86. PMID: 10364410

Negishi88: Negishi, Osamu, Ozawa, Tetsuo, Imagawa, Hiroshi (1988). "N-methyl nucleosidase from tea leaves." Agric. Biol. Chem. 52(1):169-175.

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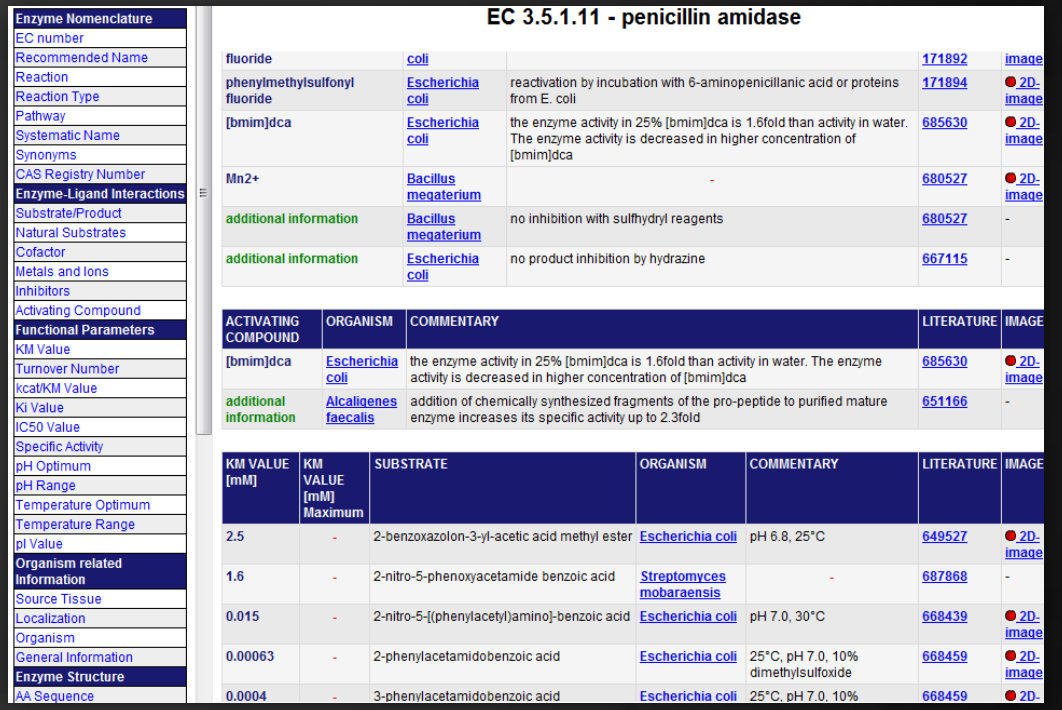
Bioinformatics for Metabolic Engineering

- BRENDA – gives hard information on enzymes

For metabolic engineering it links to MetaCyc and Kegg



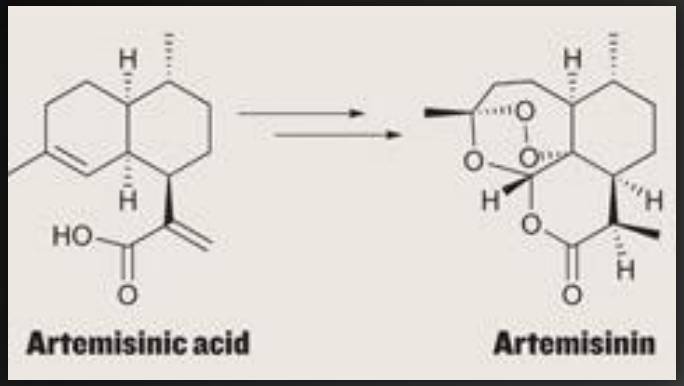
Examples of metabolic engineering by synthetic biology

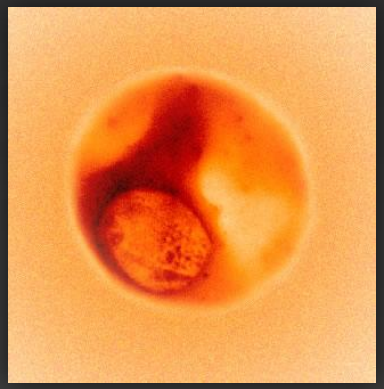
1. Yeast production of artemisinic acid for anti-malarials
   Jay Keasling Group

2. Bacterial production of iso-butanol biopetrols
   James Liao Group

3. Bacterial production of fatty acids biodiesels
   Jay Keasling Group

Artemisinin – a valuable therapeutic compound





- Artemisinin is a natural plant product from the 'sweet wormwood' plant (*A.annua*)
- It is highly effective against multi drug-resistant *Plasmodium falciparum* malaria
- Releases free-radicals in blood cells to kill off the malarial parasite
- *P.facliparum* malaria is a major problem, particularly in Africa

A.annua grows naturally only in China and Vietnam
Harvest yields of artemisinin from *A.annua* are very low

High need but low supply and high cost

Difficult and expensive to do complete chemical synthesis
Synthesis from Artemisinic Acid pre-cursor is cheap and easy

Artemisinic acid – designing its biosynthesis

Can the difficult synthesis of artemisinin be done by microbes?

A.annua converts sugars into artemisinin

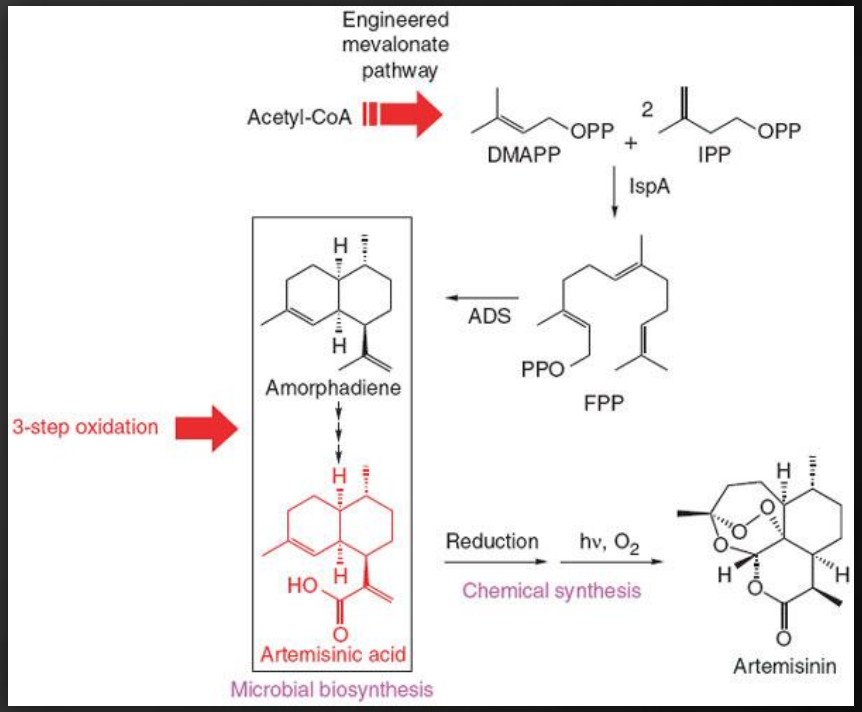
→ Import *A.annua* metabolic pathways into *E.coli* or Yeast

Arteminin from artemisinic acid can be done easily by chemistry

Artemisinic acid comes from amorphadiene
Armophadiene is made from isoprenoids

Isoprenoids are synthesised through the mevalonate pathway, already common to yeast and *E.coli*

Engineer native pathways and add *A.annua* enzymes to make artemisinic acid



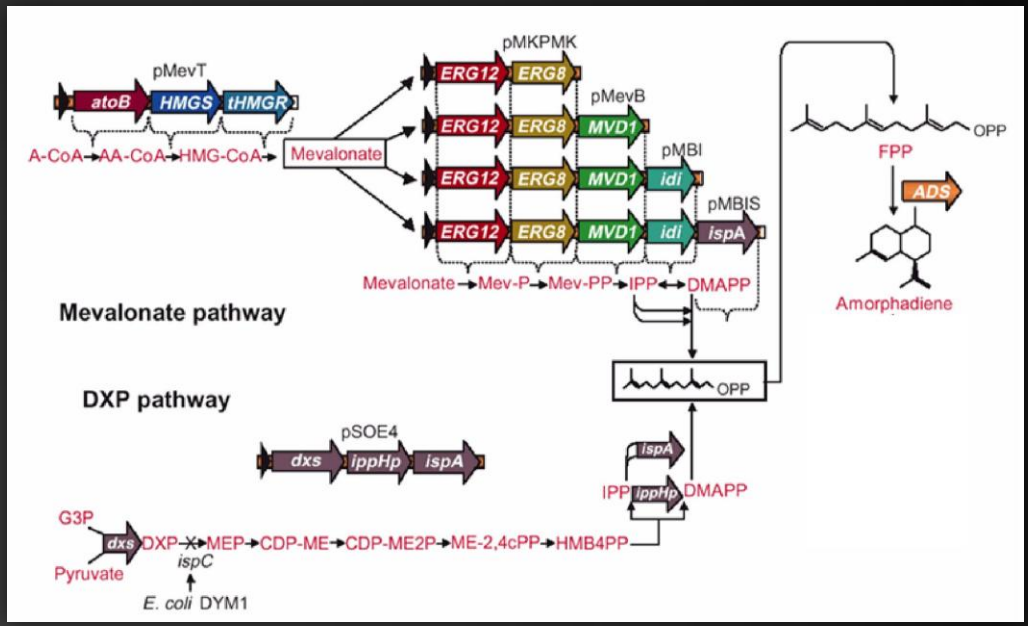
Re-engineering microbes to make amorphadiene

Yeast and *E.coli* mevalonate pathway enzymes were engineered into operon units

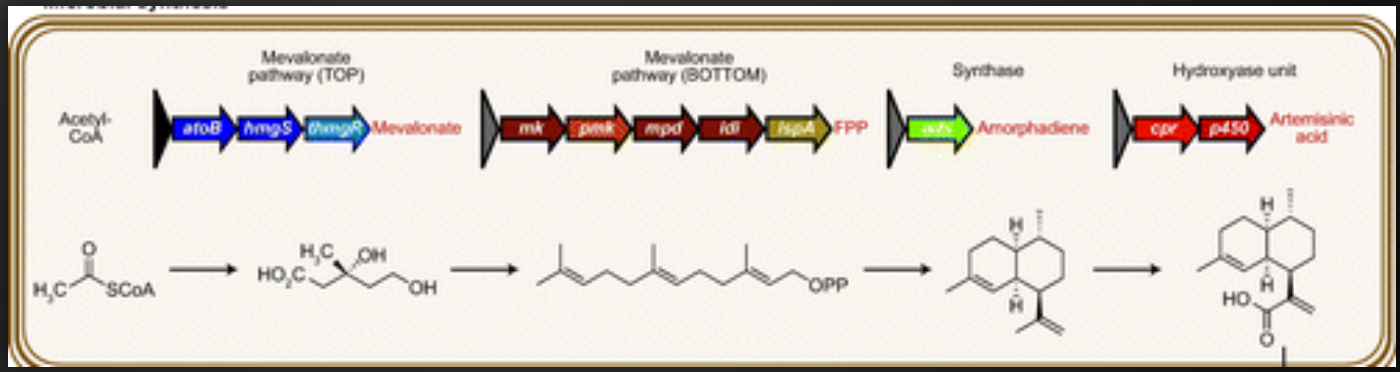
Each operon controlled by a promoter and on a plasmid

Heterologous pathways created alongside existing isoprenoid pathway

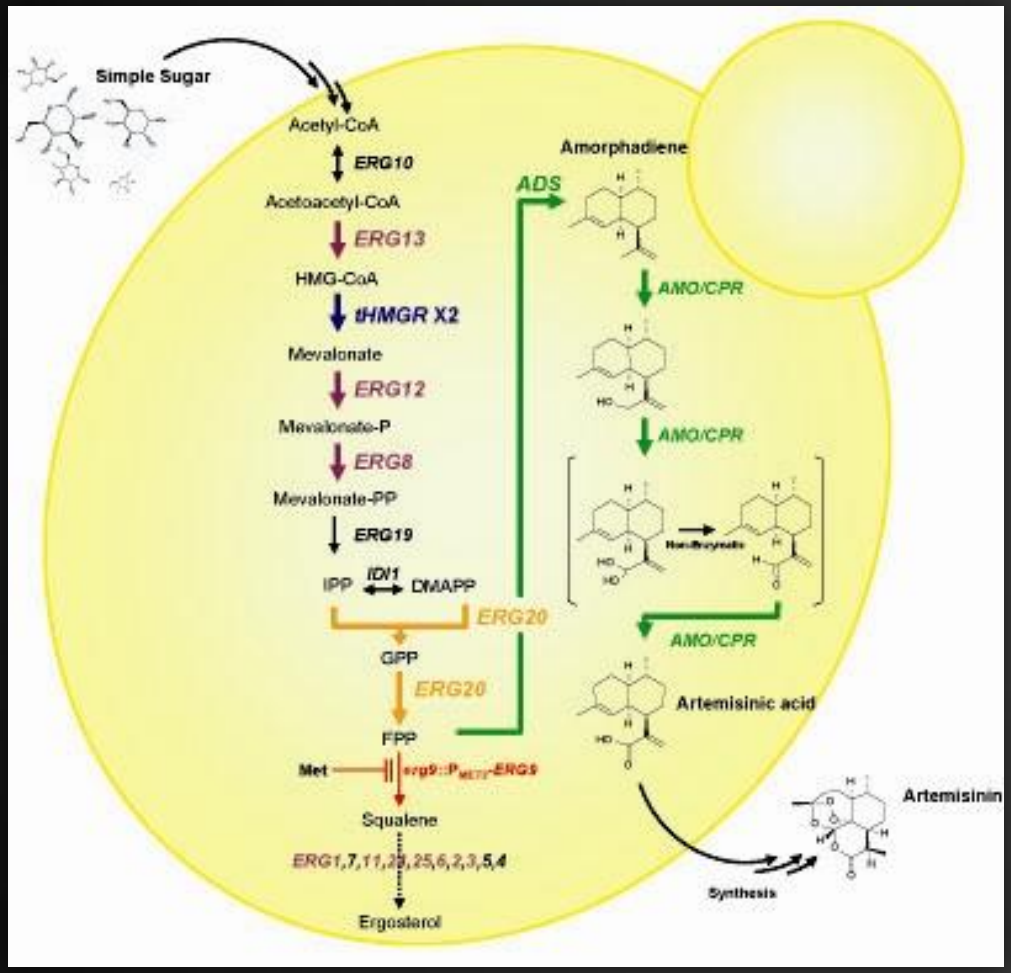
A.annua genes added to make product



Done in *E.coli* and Yeast



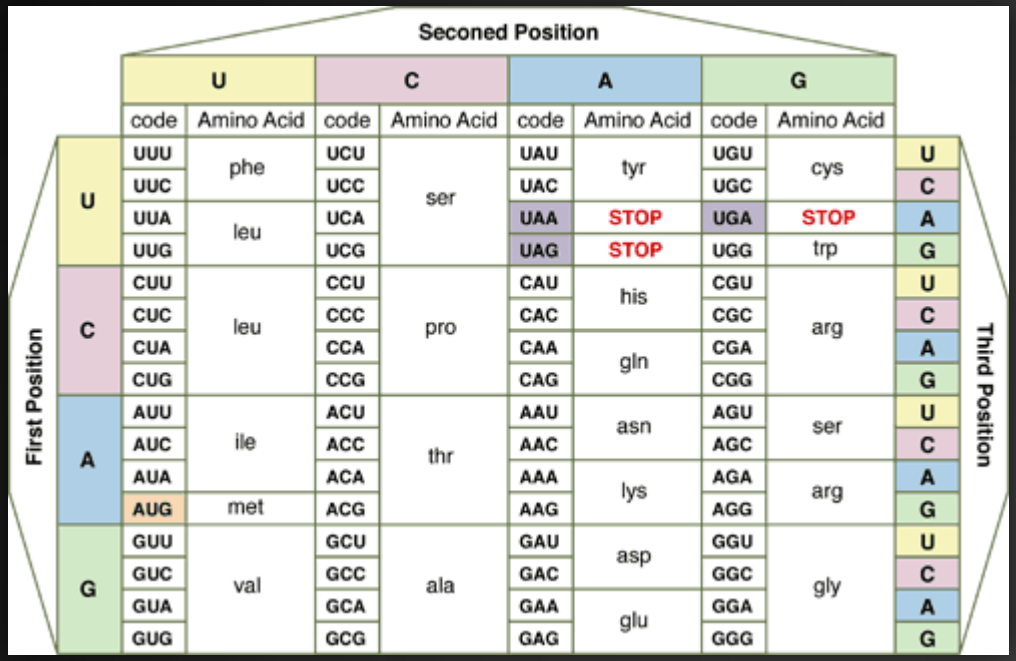
Semi-synthesis of artemisinin by engineered yeast



- Blue gene = directly up-regulated and dual-copy
- Purple gene = indirectly up-regulated by *upc2-1* – a global yeast biosynthesis regulator transcription factor
- Red line = strain has repression built-in
- Green = *A.annua* enzymes codon-optimized for yeast

DK Ro, EM Paradise et al., Nature 2006

Codon-optimisation



First Position	Seconed Position: U		Seconed Position: C		Seconed Position: A		Seconed Position: G		Third Position
	code	Amino Acid	code	Amino Acid	code	Amino Acid	code	Amino Acid	
U	UUU	phe	UCU	ser	UAU	tyr	UGU	cys	U
	UUC		UCC		UAC		UGC		C
	UUA	leu	UCA		UAA	STOP	UGA	STOP	A
	UUG		UCG		UAG	STOP	UGG	trp	G
C	CUU	leu	CCU	pro	CAU	his	CGU	arg	U
	CUC		CCC		CAC		CGC		C
	CUA		CCA		CAA	gln	CGA		A
	CUG		CCG		CAG		CGG		G
A	AUU	ile	ACU	thr	AAU	asn	AGU	ser	U
	AUC		ACC		AAC		AGC		C
	AUA		ACA		AAA	lys	AGA	arg	A
	AUG	met	ACG		AAG		AGG		G
G	GUU	val	GCU	ala	GAU	asp	GGU	gly	U
	GUC		GCC		GAC		GGC		C
	GUA		GCA		GAA	glu	GGA		A
	GUG		GCG		GAG		GGG		G

Codon	Human	Drosophila	E. coli
Arginine:			
AGA	22 %	10 %	1 %
AGG	23 %	6 %	1 %
CGA	10 %	8 %	4 %
CGC	22 %	49 %	39 %
CGG	14 %	9 %	4 %
CGU	9 %	18 %	49 %
Total number of arginine codons	2403	506	149
Total number of genes	195	46	149

Efficiency of translation is affected by the codon used
Coding sequence of a protein has an effect on how much is produced

Tuning the enzymes of the isoprenoid pathway

Key pathway to artemisinin – isoprenoids

How to up-regulate without creating bottlenecks and toxins?

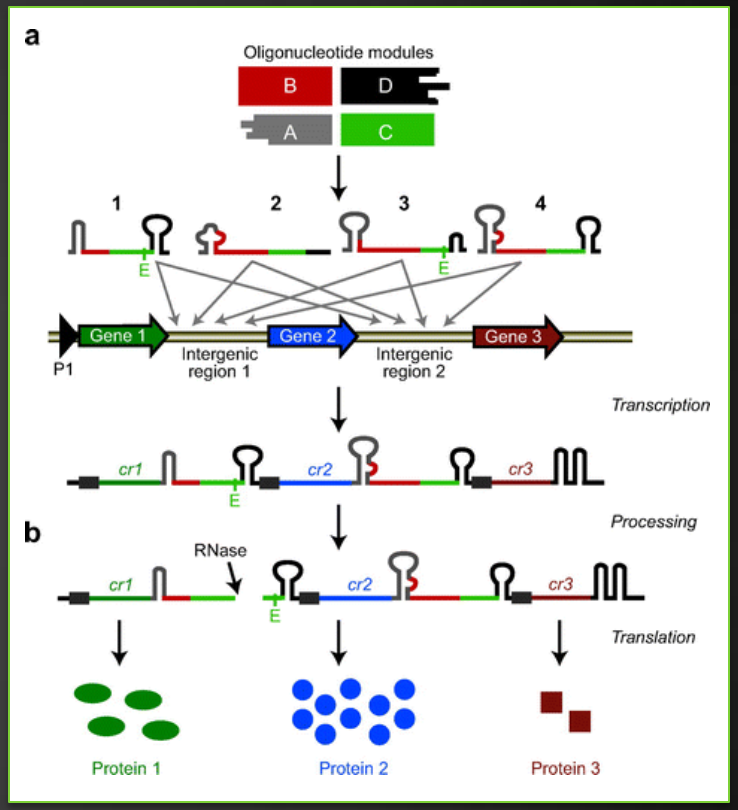
Need to tune expression of each enzyme
- Separate promoters for each?
- Directed evolution on each enzyme?

'Tunable Intergenic Regions' (TIGRs)

Modular RNA system to allow genes under the control of the same promoter to have different expression levels.

Modular = shuffling = directed evolution

Works in *E.coli* and yeast



BF Pfelger et al., Nature Biotech 2006

Beyond artemisinic acid – the value of isoprenoids

Gates Foundation, Sanofi-Aventis - Africa

Full synthesis to Artemisinin

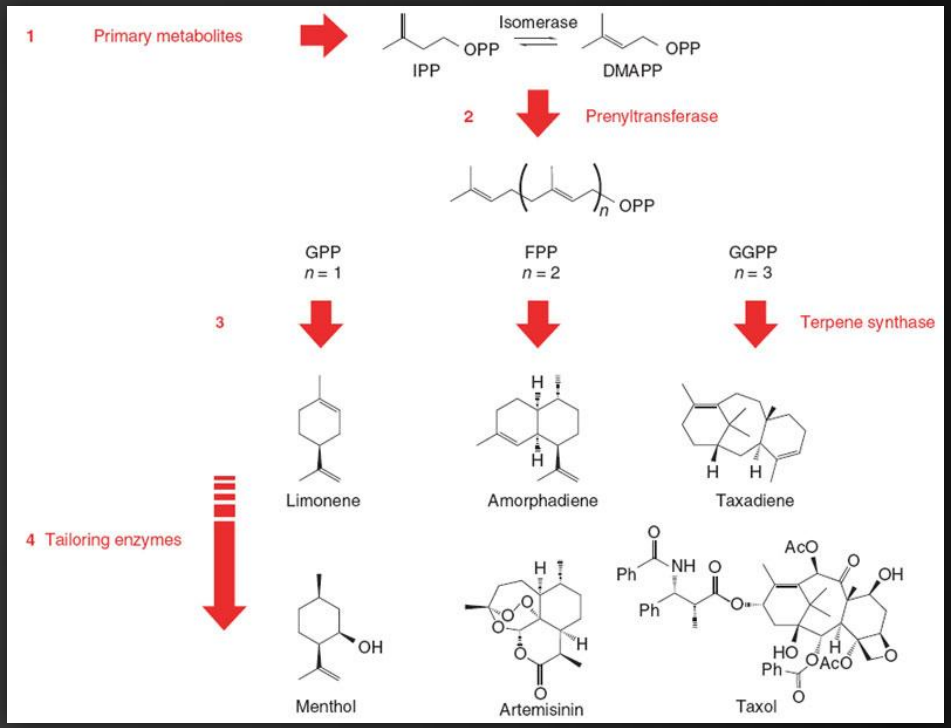
Improved synthesis:

every ATP counts

Other plant products – Taxol

Industry - Amyris

(biodiesels, aviation fuels and more)



Increased synthesis of mevalonate with scaffolds

Mevalonate synthesis in *E.coli*

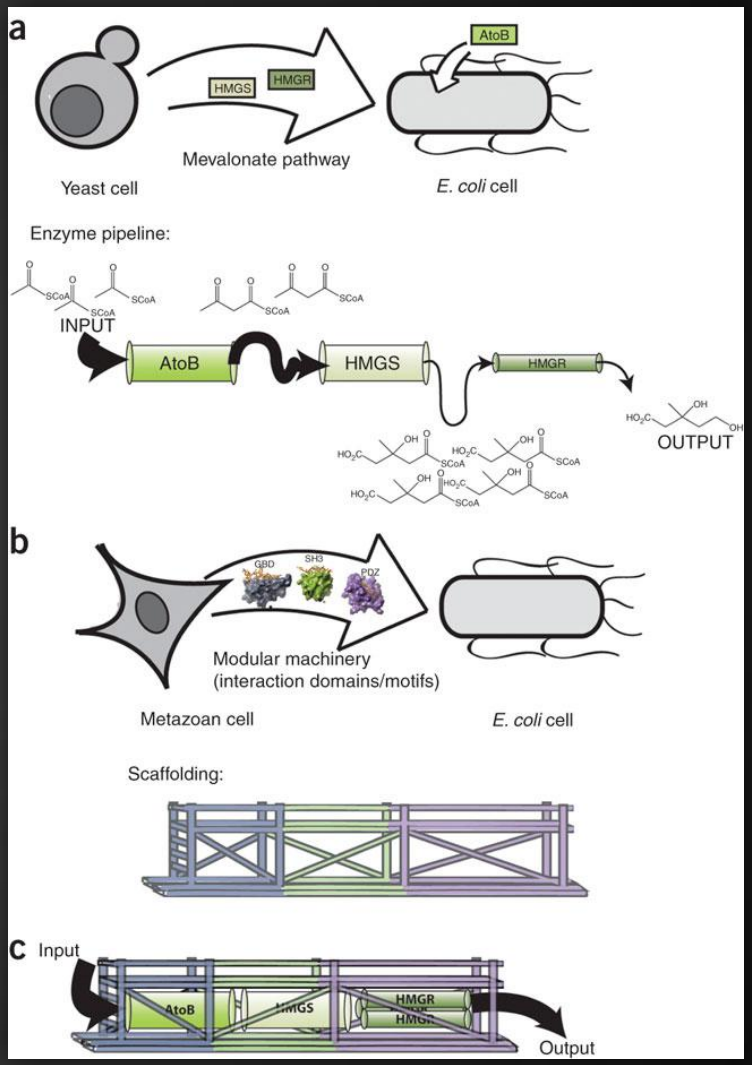
Create a 3-enzyme mevalonate pathway:
1 x *E.coli* , 2 x codon-optimized Yeast

Fuse each enzyme to a binding domain
Co-express a modular yeast scaffold protein

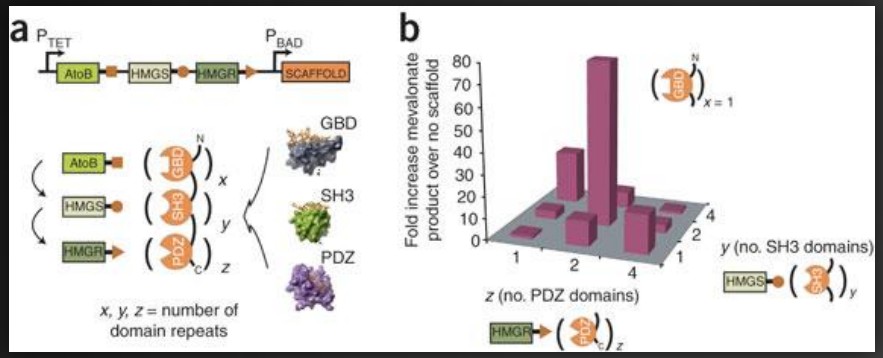
Scaffold protein binds all 3 enzymes next to each other

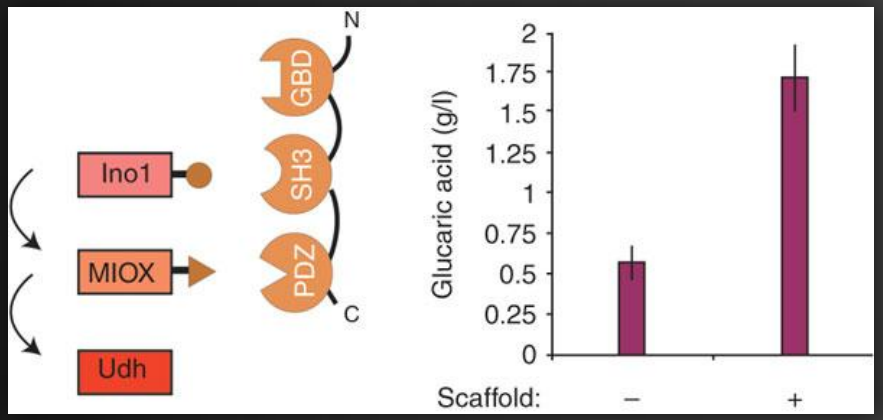
1. Increases pathway flux
2. Reduces toxic intermediate build-up
3. Prevents negative feedback inhibition from metabolite accumulation

Dueber et al., Nature Biotech 2009

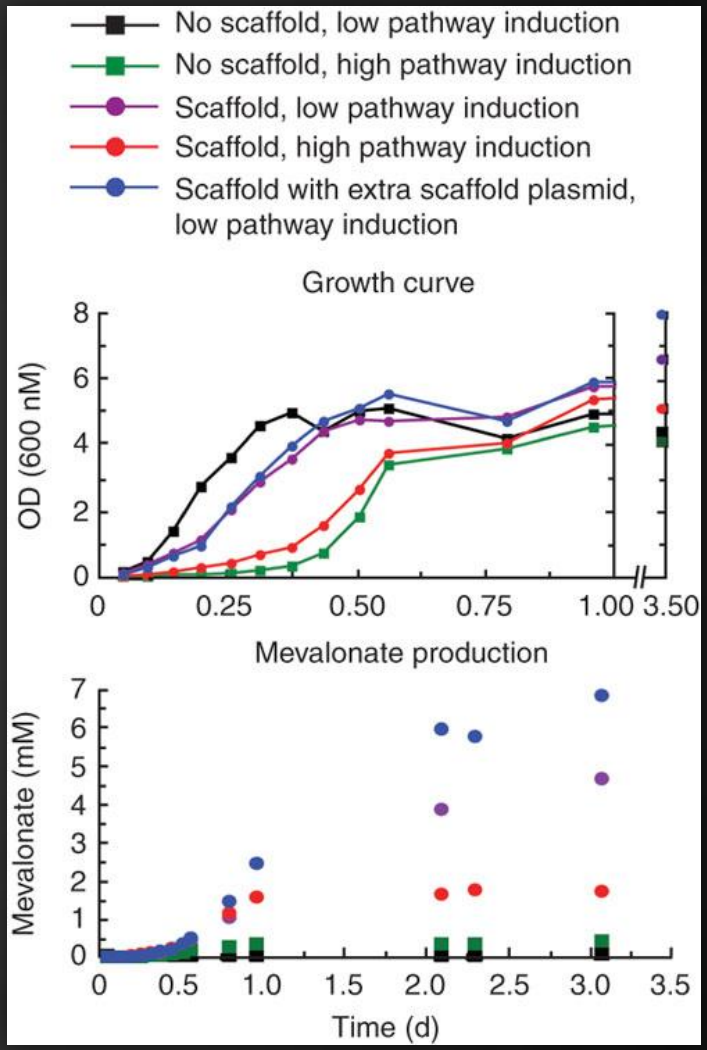


Mevalonate and glucaric acid synthesis with scaffolds



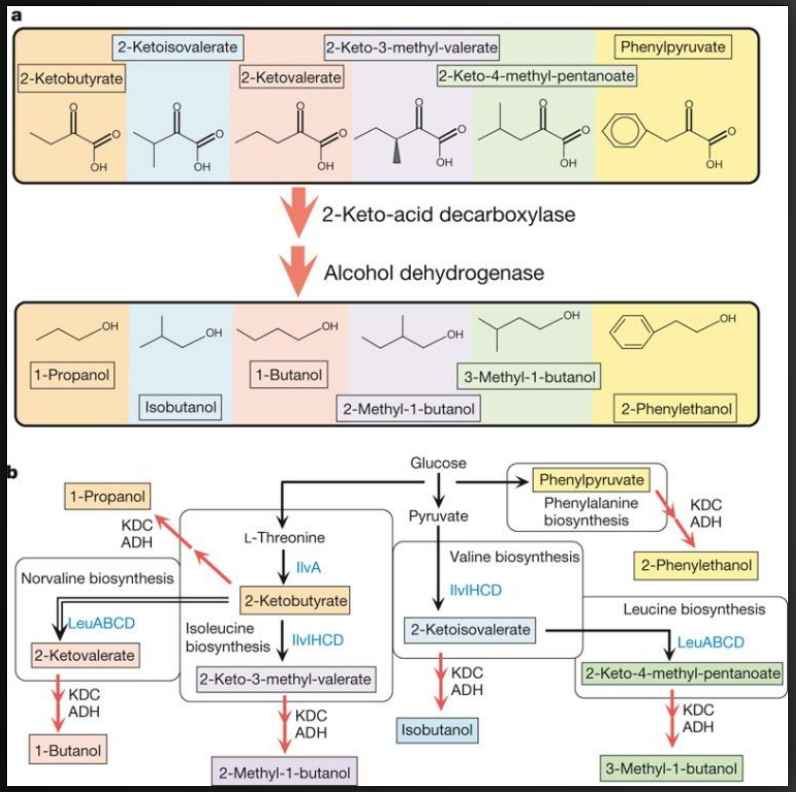


Dueber et al., Nature Biotech 2009



Petrol from *E.coli* – engineering iso-butanol production

Iso-butanol can be a direct replacement for petrol
Naturally synthesized by some strains of *Clostridium* – one of the oldest biotechnologies
Not produced naturally by any non-fermenting cells growing on glucose



S Atsumi et al., Nature 2008

- Use keto-acid pathways (amino acids synthesis)
- Overexpress entire pathway operons on plasmids
- Delete (knock-out) by-product forming enzymes

- Add a variety of different decarboxylases and dehydrogenases and look for product yields: *E.coli*, Yeast, *Clostridium*, *L.lactis*, *B.subtilis*

Table 1 | Alcohol production with KDC and ADH in *E. coli*

Product (μM)	KDC/plasmid				
	Kivd/pSA55	Aro10/pSA56	Pdc6/pSA49	Thi3/pSA57	Pdc (*C. acetobutylicum*)/pSA58
1-Propanol	520	290	125	ND	ND
Isobutanol	5,242	2,094	260	ND	75
1-Butanol	220	95	ND	ND	ND
2-Methyl-1-butanol	766	652	56	ND	ND
3-Methyl-1-butanol	1,495	1,099	92	ND	ND
2-Phenylethanol	324	469	ND	ND	175

The strain was JCL16 with various *kdc* genes and *S. cerevisiae ADH2* expressed from plasmids. Culture was grown in M9 medium with 0.2 M glucose plus 0.1 mM IPTG at 30 °C for 40 h. These products were identified by GC–MS and quantified by GC–FID (see Methods). ND, not detectable.

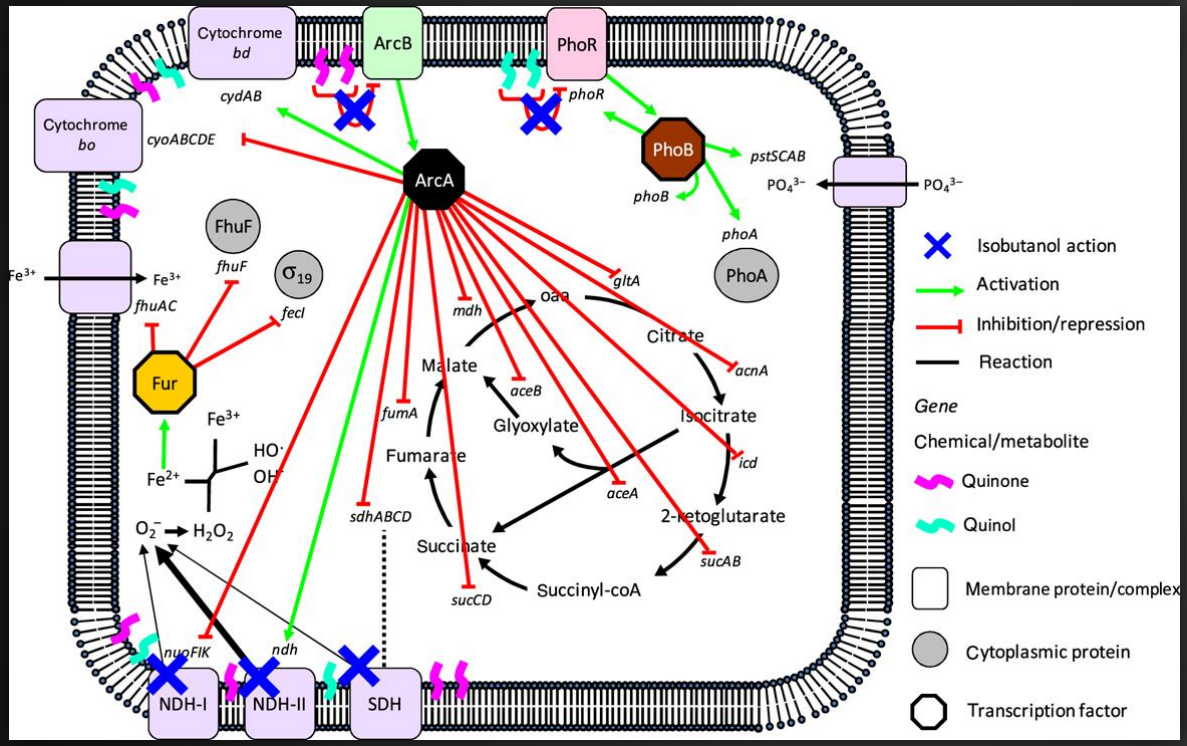
Table 2 | Alcohol production with the supply of 2-keto acids

Product (μM)	2-Ketobutyrate	2-Keto-isovalerate	2-Ketovalerate	2-Keto-3-methyl-valerate	2-Keto-4-methyl-pentanoate	Phenylpyruvate
1-Propanol	2,138	ND	ND	ND	ND	8
Isobutanol	98	10,016	ND	ND	ND	64
1-Butanol	492	ND	3,926	ND	ND	23
2-Methyl-1-butanol	1,315	ND	ND	5,284	ND	ND
3-Methyl-1-butanol	ND	ND	52	ND	3,756	105
2-Phenylethanol	26	109	66	ND	ND	7,269

Strains and culture conditions are the same as described in Table 1. A total of 8 g l^{-1} of 2-keto acids was added, except for 2-ketovalerate, where 1 g l^{-1} was added because of its toxicity. ND, not detectable.

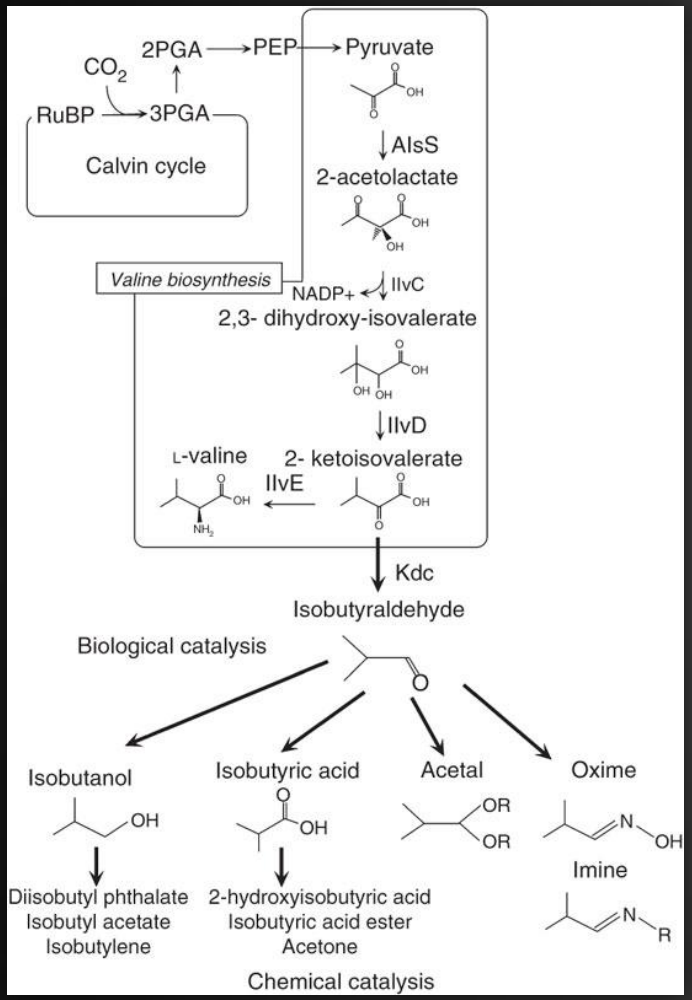
Petrol from *E.coli* keto-acids – systems biology analysis

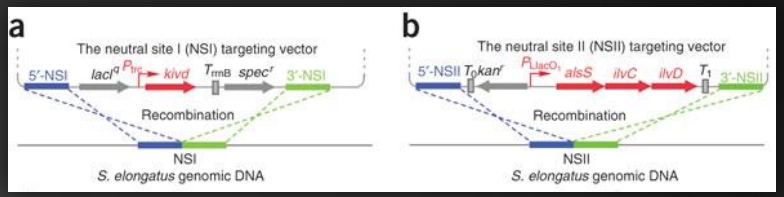
- Network analysis follow-up study
- How does gene expression change when iso-butanol production is added?
- Do expression array studies and build a network map to indentify master controllers

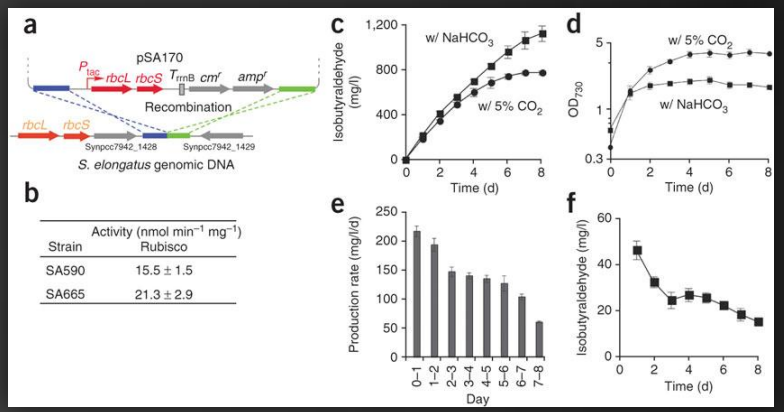


MP Brynildsen and JC Liao, Mol Sys Biol 2009

Petrol from CO_2 – extending iso-butanol production







S Atsumi et al., Nature Biotech 2009

Synechococcus elongatus – cyanobacteria

- Overexpress related Rubisco enzymes
- Add keto-acid genes from *E.coli, B.subtilis, L.lactis*
- Chromosomal integration required

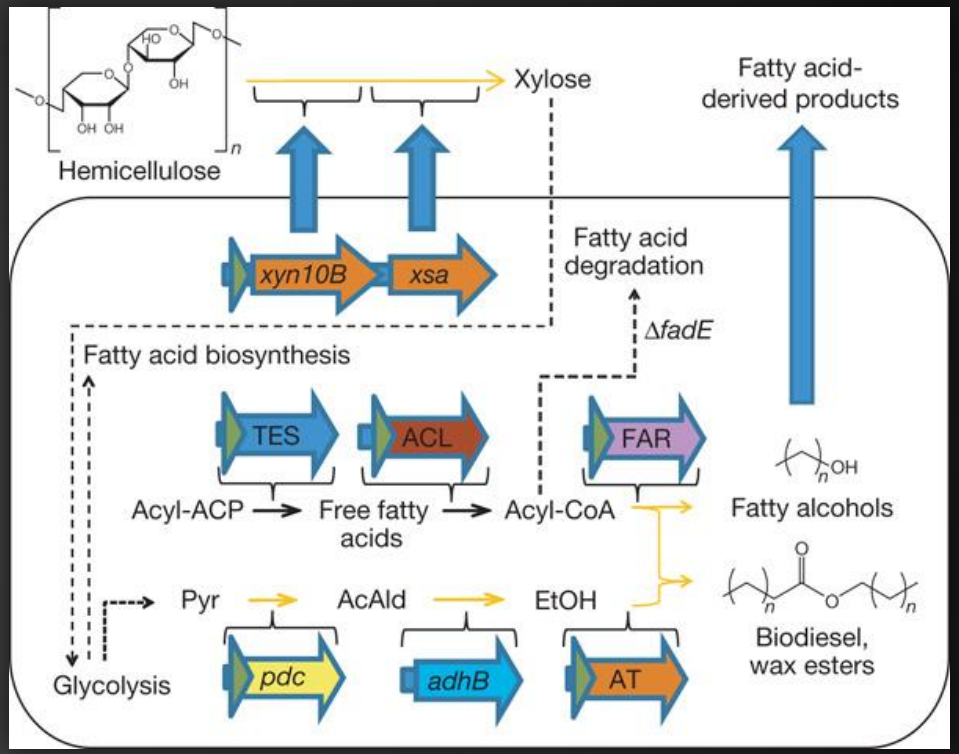
Fatty-acid biodiesel production in plant-digesting *E.coli*

1. Remove feedback inhibition from native fatty acid synthesis
   - Overexpress and free thioesterase
   - Overexpress 1st step of degradation

2. Prevent fatty acid degradation
   - Delete 2nd step of pathway from the cell genome (*fadE*)

3. Convert fatty acids into fatty alcohols
   - Express codon-optimized mouse FAR enzyme

4. Assemble new pathway to synthesize ethanol
   - Express *Zymomonas pdc* and *adhB*



EJ Steen and Y Kang et al., Nature 2010

Fatty-acid biodiesel production in plant-digesting *E.coli*

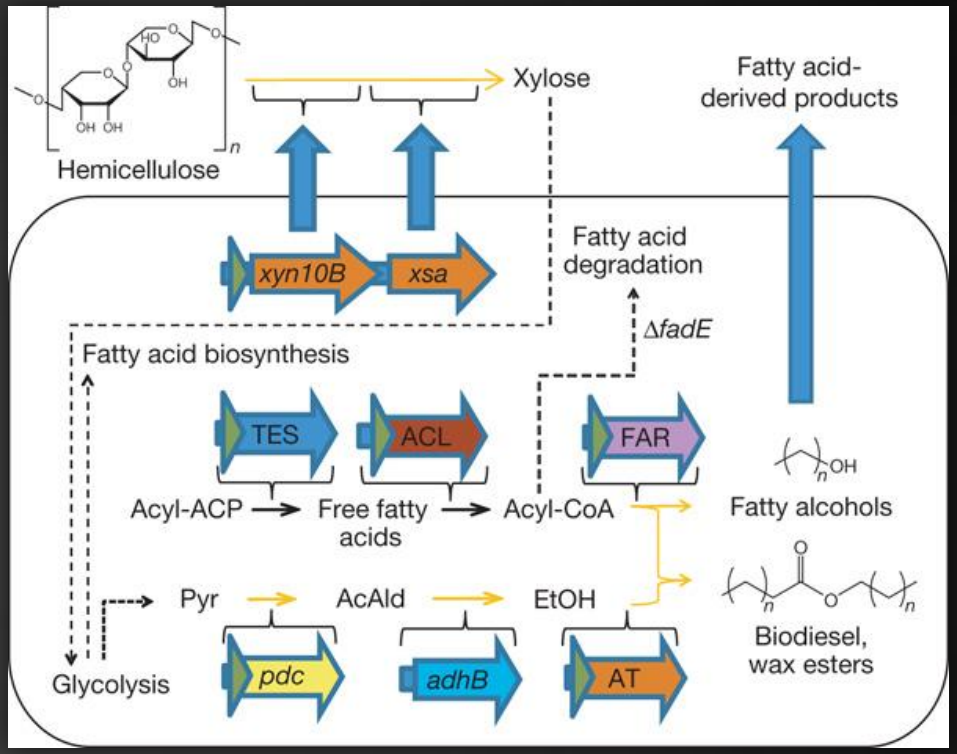
5. Combine ethanol and fatty acids and alcohols to get biodiesels, wax esters
- Express *Acinetobacter* AT gene

6. Use hemicellulose as a food source
- Fuse codon-optimized cellulases from *Clostridium* and *Bacteroides* to cell surface proteins

Fatty products secrete from the cells

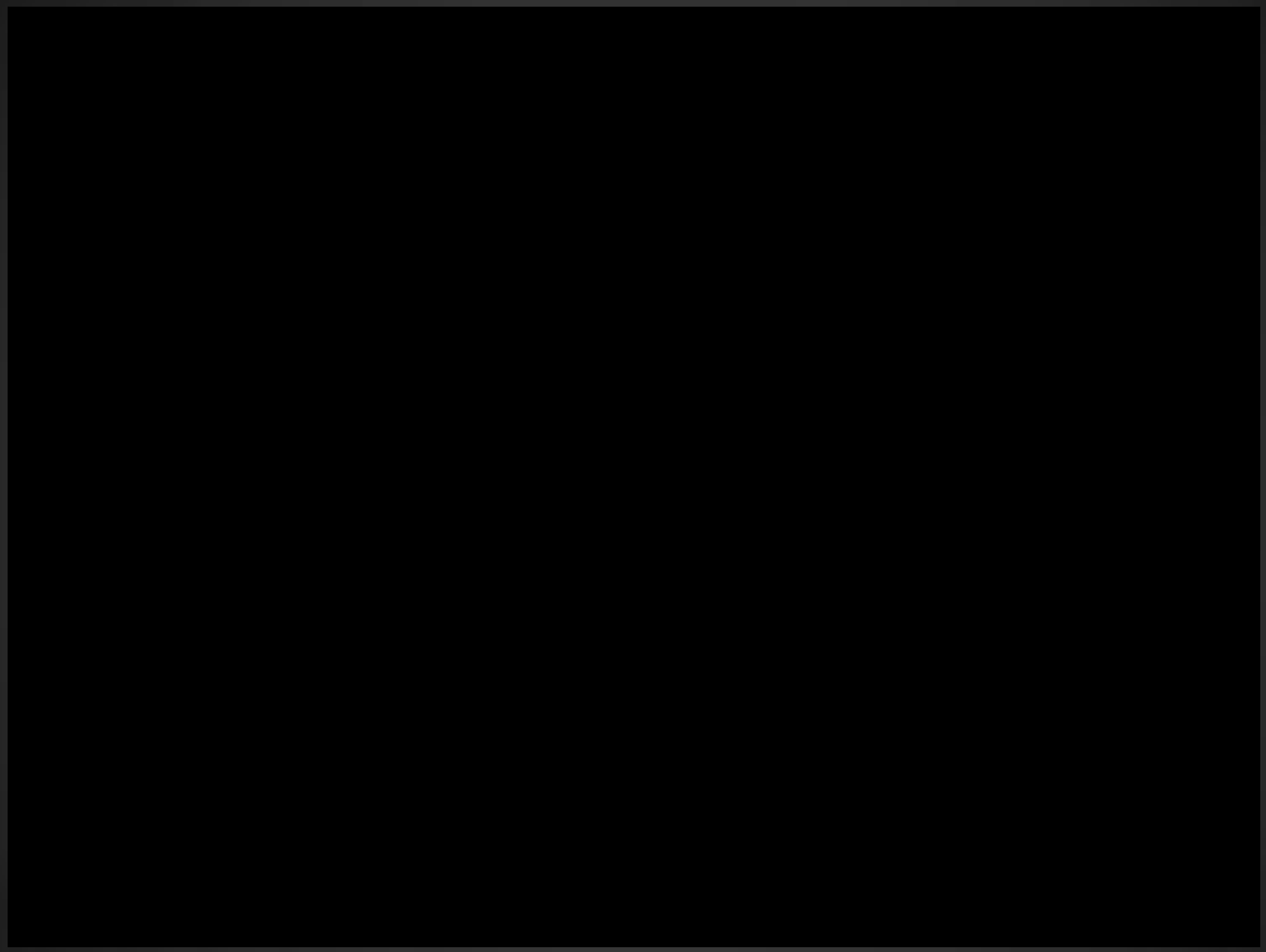
Swapping thioesterase (TES) from different organisms gives different fatty acid product lengths

All done by adding plasmids with genes with strong, regulated promoters



EJ Steen and Y Kang et al., Nature 2010

Fatty-acid biodiesel production in plant-digesting *E.coli*



BBC4 "The Cell" episode 3, 2009

Summary of synthetic biology used so far

- Up-regulate enzymes and pathways by cloning techniques
  - strong promoters, plasmids, codon-optimisation
- Add foreign enzymes from all across nature
  - create heterologous pathways, test out different versions of enzymes
- Co-ordinated expression of many enzymes
  - express genes from same regulated promoter
  - tune relative levels in an operon with TIGRs / IRES / regulation units
- Knock-out competing pathways and enzymes by modifying genomic DNA
- Use mutation/shuffling and selection to evolve increase yields
- Co-localize pathway enzymes on a scaffold to increase flux to product
- Combine FBA and systems biology to determine global regulators

The future for metabolic engineering

- The minimal cell – "tailor-made" cell chassis (JC Venter)
- Industrial metabolic engineering – e.g. LS9, Amyris
- Yeast – cytochromes, post-translation protein modifications
- Algae – biofuel from sunlight, hydrogen, even olive oil



- Automation of flux balance in pathways by shuffle/evolution
- Self-regulation built in to pathways
- Predictive network models linking genome to metabolic flux
- Consortia of engineered microbes growing with different roles

What you should now know and read up on!

You could get exam questions on...

1. The complexity and diversity of metabolism and how these are a challenge to engineering
2. The synthetic biology tools that can be used to overcome these challenges in metabolic engineering
3. The use of modeling, bioinformatics and systems biology in metabolic engineering
4. Examples of high-value products that could be made by metabolic engineering
5. How microbes have been engineered to help in the synthesis of artemisinin
6. Production of biofuels by engineered microbes (x2)

What you would synthesise, why and how?

Reading – useful reviews and perspectives

Toward engineering synthetic microbial metabolism – GH McArthur and SS Fong
Journal of biomedicine & biotechnology, Vol. 2010 (2010)

Synthetic Biology for Synthetic Chemistry – JD Keasling
ACS Chemical Biology, Vol. 3, No. 1. (1 January 2008), pp. 64-76.

Engineering for biofuels: exploiting innate microbial capacity or importing biosynthetic potential? – H Alper and G Stephanopoulos
Nature Reviews Microbiology, Vol. 7, No. 10. (01 October 2009), pp. 715-723.

Chemical synthesis using synthetic biology. – JM Carothers et al.
Current opinion in biotechnology, Vol. 20, No. 4. (31 August 2009), pp. 498-503.

Synthetic Metabolism: Engineering Biology at the Protein and Pathway Scales – CH Martin et al
Chemistry & Biology, Vol. 16, No. 3. (27 March 2009), pp. 277-286.

Advances in flux balance analysis – KJ Kauffman et al.
Current Opinion in Biotech, Vol. 14, No. 5. (October 2003), pp. 491-496.